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BACTERIAL STRESS PROTEINS

Abstract:

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(54) Title: BACTERIAL STRESS PROTEINS (57) Abstract A bacterial stress protein is described which is obtainable from Gram-positive bacteria, such as strains of <i>Corynebacterium jeikeium</i> . Also described are antibodies capable of recognising the stress protein and these, as well as the stress protein, provide separate means for the diagnosis and/or treatment of bacterial, particularly Coryneform infections.		

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BACTERIAL STRESS PROTEINS

Field of the Invention

This invention relates to bacterial stress proteins, to inhibitors thereof and to their use in medicine and for diagnosis.

Background to the Invention

Environmental stress can induce an increase in the rate of synthesis of so-called heat shock, or stress, proteins in both procaryotic and eucaryotic cells [see for example Schlesinger et al (eds) in Heat Shock from Bacteria to Man, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1972)]. Although the function of stress proteins has yet to be finally resolved, some have been reported to participate in assembly and structural stabilisation of certain cellular and viral proteins, and their presence at high concentration may have an additional stabilising effect during exposure to adverse conditions.

Many pathogenic organisms have been shown to produce stress proteins [see for example Young D., et al, Proc. Natl. Acad. Sci. USA, 85, 4267-4270 (1988)]. The proteins are thought to be produced in response to the stress of infection to help protect the invading pathogen. Thus, for example, the ability to produce stress proteins has been implicated in the survival of bacterial pathogens within macrophages [Christmas, M.F., et al, Cell, 41, 753-762 (1985) and Morgan R.W., et al, Proc. Natl. Acad. Sci. U.S.A, 83, 8059-8063 (1986)].

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It has been suggested that the presence of stress proteins in a variety of human pathogens indicates that the stress response is a general component of infections, and that stress proteins should be considered among candidates for subunit vaccines [Young, D. et al *ibid*].

The Coryneform bacteria are common inhabitants of healthy human skin and mucous membranes. Some are known pathogens, for example the type species Corynebacterium diphtheriae causes diphtheria in man. Another species, Corynebacterium jeikeium causes septicaemias in neutropenic patients [see for example, Hande, K.R., et al, *Ann. Intern. Med.*, 85, 423-426 (1976)], and C. jeikeium infection has been associated with endocarditis [Jackman, P.J., et al, *Syst. Appl. Microbial.*, 9, (1-2), 83-90 (1987)]. These C. jeikeium species are characterised by being catalase-positive, oxidase-negative, fermentative Gram positive aerobic rods which do not reduce nitrate [Riley, P.S., et al, *J. Clin. Microbial.*, 9, 418-424, (1979)]. They differ from other coryneforms by being highly resistant to antibiotics, including ampicillin, cephalothin, chloramphenicol, erythromycin, gentamicin, penicillin G, streptomycin and tetracycline [Jackman, P.J., Pelczynska, S., *J. Gen. Microbial.*, 132, (Pt.7), 1911-1915 (1986)].

Conventional therapy for a C. jeikeium infection is systemic vancomycin, which is potentially nephrotoxic. This mitigates against its blind use in pyrexial neutropenic patients, so that it has become important to develop a marker of C. jeikeium infection. There is also a need for a method of treating C. jeikeium infection, particularly in neutropenic patients, which avoids the damaging side effects associated with conventional therapy.

Summary of the Invention

We have now found that recovery from C.jejikeium septicaemia in humans is associated with the production of antibodies of both IgM and IgG classes against a protein of apparent molecular weight 52Kd. Further tests using antibodies raised against a known fungal stress protein have shown that the 52Kd protein is a breakdown product of an 86Kd antigen analogous to the 90Kd heat shock protein of Candida albicans. We have used this discovery to develop means for the improved diagnosis and treatment of *Corynebacterium* infections and diseases caused by related Gram positive bacteria.

Thus according to one aspect of the invention we provide a bacterial stress protein having an apparent molecular weight of around 86Kd or a fragment or an analogue thereof.

The term "apparent molecular weight" as used herein is intended to mean the apparent molecular weight as determined by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis using molecular weight markers supplied by Amersham International (Prestained Rainbow Markers, code RPN.756).

The stress protein according to the invention may be of bacterial origin and may be obtainable, for example, from Gram-positive bacterial strains. Particular strains include those belonging to the genus *Corynebacterium*, for example strains of *Corynebacterium diphtheriae* or, in particular, *Corynebacterium jeikeium*, and related *Coryneform* species.

The stress protein, fragment or analogue thereof according to the invention may be a recombinant protein, fragment or analogue, i.e. a protein, fragment or analogue which has been produced using recombinant DNA techniques.

A fragment of a stress protein according to the invention may be any shorter breakdown product of the protein or an analogue thereof. Particular fragments include those with apparent molecular weights of around 52Kd or 50Kd.

Analogues of a stress protein according to the invention include those proteins wherein one or more amino acids of the protein is replaced by another amino acid, providing that the overall functionality of the protein is conserved.

A stress protein according to the invention may be obtained in a purified form, and thus according to a further aspect of the invention we provide a substantially pure bacterial stress protein having an apparent molecular weight of around 86Kd or a fragment or an analogue thereof.

The term substantially pure is intended to mean that the stress protein according to the invention is free from other proteins of bacterial origin. In the various aspects of the invention described hereinafter it is to be understood that a reference to the bacterial stress protein also includes substantially pure preparations of the protein.

A stress protein according to the invention may be further characterised by one or more of the following features:

- (1) it is an immunodominant conserved antigen;

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- (2) patients with C.jejikeium septicaemia on recovery have antibody to a 52Kd breakdown product of the stress protein; and
- (3) patients with C.jejikeium endocarditis have antibody to a 52kd breakdown product of the stress protein;
- (4) it cross-reacts with a peptide antigen KVIRKNIVKKMIE using mouse monoclonal antibody against the peptide antigen; and
- (5) cross-reaction of the stress protein and mouse monoclonal antibody against the peptide antigen KVIRKNIVKKMIE is neutralised by peptide KVIRKNIVKKMIE.

Throughout this document peptides are identified using a single letter to represent each separate amino acid. Each letter is the conventional single letter symbol used for amino acids.

A stress protein according to the invention has a number of uses. Thus, for example, the protein may form the basis of a diagnostic test for bacterial infection, for example an immunological test such as an enzyme-linked immunosorbent assay, a radioimmunoassay or a latex agglutination assay, essentially to determine whether antibodies specific for the protein are present in a host organism. The test may be generally performed by contacting body fluid from the host with the stress protein and detecting any complexed material.

In another use, the stress protein according to the invention may be employed, using conventional techniques,

for screening to obtain activity inhibiting agents for use in the treatment of bacterial infections. Such a screening method forms a further aspect of the invention.

In a further use, the stress protein according to the invention is particularly well suited for the generation of antibodies. Thus according to a further aspect of the invention we provide a bacterial stress protein having an apparent molecular weight of 86Kd or a fragment or an analogue thereof, for use as an immunogen.

Standard immunological techniques may be employed with the stress protein in order to use it as an immunogen. Thus, for example, any suitable host may be injected with the protein and the serum collected to yield the desired polyclonal anti-stress protein antibody after purification and/or concentration. Prior to injection of the host the stress protein may be formulated in a suitable vehicle and thus according to a further aspect of the invention we provide a composition comprising a fungal stress protein having an apparent molecular weight of 86Kd or a fragment or an analogue thereof together with one or more pharmaceutically acceptable excipients.

For purification of any anti-stress protein antibody, use may be made of affinity chromatography employing an immobilised stress protein of the invention as the affinity medium. Thus according to another aspect of the invention we provide a bacterial stress protein having an apparent molecular weight of 86Kd or a fragment or an analogue thereof, covalently bound to an insoluble support.

The use of the stress proteins according to the invention as immunogens for the production of antibodies generates

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one type of inhibitor of the action of the protein. Generally, inhibitors of the stress proteins are potentially useful in the diagnosis, and in particular the treatment, of bacterial infections and provide a further feature of the invention. Inhibitors include any antagonists of the action of the stress proteins or agents which prevent their production, and in particular include those which may be used in the treatment of bacterial infections. Suitable inhibitors include for example pharmaceutical reagents, including antibodies, and chemical analogues of the stress proteins to antagonise the action of the stress protein, and anti-sense RNA and DNA oligonucleotide analogues to prevent production of the stress protein. Suitable inhibitors may be determined using appropriate screens, for example by measuring the ability of a potential inhibitor to antagonise the action of, or prevent the production of a stress protein according to the invention or a fragment or an analogue thereof.

According to a further aspect of the invention we provide an inhibitor of a bacterial stress protein, said protein having an apparent molecular weight of 86Kd or a fragment or an analogue thereof, for use in the diagnosis or treatment of bacterial infections.

Inhibitors may be used either alone or where appropriate in combination with other pharmaceutical agents, for example, other anti-bacterial agents or anti-fungal agents.

One particularly useful group of inhibitors according to this aspect of the invention are antibodies capable of recognising and binding to the stress proteins.

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Thus, according to yet another aspect of the invention we provide an antibody specific for one or more epitopes of a bacterial stress protein having an apparent molecular weight of 86Kd or a fragment or an analogue thereof, for use in the diagnosis or treatment of bacterial infections.

The antibody may be a whole antibody or an antigen binding fragment thereof and may in general belong to any immunoglobulin class. Thus, for example, it may be an immunoglobulin M antibody or, in particular, an immunoglobulin G antibody. The antibody or fragment may be of animal, for example mammalian origin and may be for example of murine, rat or human origin. It may be a natural antibody or a fragment thereof, or, if desired, a recombinant antibody or antibody fragment, i.e. an antibody or antibody fragment which has been produced using recombinant DNA techniques.

Particular recombinant antibodies or antibody fragments include, (1) those having an antigen binding site at least part of which is derived from a different antibody, for example those in which the hypervariable or complementarity determining regions of one antibody have been grafted into the variable framework regions of a second, different antibody (as described in European Patent Specification No. 239400); (2) recombinant antibodies or fragments wherein non-variable domain sequences have been substituted by non-variable domain sequences from other, different antibodies (as described in European Patent Specifications Nos. 120694, 125020, 171496, 173494 and 194276); or (3) recombinant antibodies or fragments possessing substantially the structure of a natural immunoglobulin but wherein the hinge region has a different number of cysteine residues from that found in

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the natural immunoglobulin, or wherein one or more cysteine residues in a surface pocket of the recombinant antibody or fragment is in the place of another amino acid residue present in the natural immunoglobulin (as described in International Patent Specifications Nos. WO89/01974 and WO89/01782 respectively).

The antibody or antibody fragment may be of polyclonal, or preferably, monoclonal origin. It may be specific for a number of epitopes associated with the stress protein but it is preferably specific for one.

Antigen binding antibody fragments include for example fragments derived by enzymatic cleavage of a whole antibody, such as F(ab')₂, Fab', Fab or Fv fragments, or fragments obtained by recombinant DNA techniques, for example as described in International Patent Specification No. WO89/02465, as well as single chain antibodies, e.g. single chain Fvs.

The antibody or fragment may be cross-linked, for example as described in European Patent Specification No. 385601. Antibody or antibody fragment molecules may also be cross-linked to yield multimeric forms [for example IgG molecules may be cross-linked to form trimeric, tetrameric or larger multimeric structures], using conventional cross-linking approaches, for example by cross-linking amino acid side chains present in the molecules, and containing an amino, sulphydryl or other functional group, using a cross-linking reagent.

The antibodies according to the invention may be prepared using well-known immunological techniques employing the stress protein as antigen. Thus, for example, any

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suitable host may be injected with the stress protein and the serum collected to yield the desired polyclonal antibody after appropriate purification and/or concentration (for example by affinity chromatography using the immobilised stress protein as the affinity medium). Alternatively splenocytes or lymphocytes may be recovered from the stress protein injected host and immortalised using for example the method of Kohler et al, Eur. J. Immunol. 6, 511, (1976), the resulting cells being segregated to obtain a single genetic line producing monoclonal anti-fungal stress proteins. Antibody fragments may be produced using conventional techniques, for example by enzymatic digestion, e.g. with pepsin [Parham, J. Immunol, 131, 2895 (1983)] or papain [Lamoyi and Nisonoff, J. Immunol. Meth., 56, 235, (1983)]. Where it is desired to produce recombinant antibodies according to the invention these may be produced using for example the methods described in European Patent Specifications Nos. 171496, 173494, 194276 and 239400.

Antibodies according to the invention may be labelled with a detectable label or may be conjugated with an effector molecule for example a drug e.g. an anti-bacterial agent, conventional procedures and the invention extends to such labelled antibodies or antibody conjugates.

The antibodies according to the invention have a diagnostic and/or therapeutic use. Thus for diagnostic use the antibodies may be employed to detect whether the stress protein is present in a host organism, to confirm whether the host has a particular bacterial infection, for example an infection due to a Gram-positive organism, especially a Coryneform organism, particularly C.diphtheriae or C.jejkeium, for example in the diagnosis

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of septicaemias or endocarditis, and/or to monitor the progress of therapeutic treatment of such infections. Diagnostic methods of this type form a further aspect of the invention and may generally employ standard techniques, for example immunological methods such as enzyme-linked immunosorbent methods, radioimmuno-methods, latex agglutination methods or immunoblotting methods. Such tests generally bring into contact the antibody and host body fluid and detect any resulting complexed antibody.

Antibodies according to the invention also have a therapeutic use in the treatment of bacterial infections, for example those due to Gram-positive bacteria, especially Coryneform organisms as just described, and may be used alone or conjugated to an effector molecule, [in the latter case to target the effector molecule, e.g. an anti-bacterial agent to the infecting organism], optionally in combination with other pharmaceutical agents, such as other antibacterial or anti-fungal agents. For therapeutic use the antibody may be formulated in accordance with conventional procedures, for example with a pharmaceutically acceptable carrier or excipient, e.g. isotonic saline for administration at an appropriate dosage, depending on the nature of the infection to be treated and the age and condition of the patient.

A particularly useful antibody according to this aspect of the invention is that which recognises the peptide KVIRKNIVKKMIE and according to a further aspect of the invention we provide an antibody capable of recognising the peptide KVIRKNIVKKMIE or an analogue thereof for use in the treatment or diagnosis of bacterial infection.

If desired, mixtures of antibodies may be used for diagnosis or treatment, for example mixtures of two or more antibodies recognising different epitopes of a bacterial stress protein according to the invention, and/or mixtures of antibodies of a different class, e.g. mixtures of IgG and IgM antibodies recognising the same or different epitope(s) of a bacterial stress protein of the invention.

The stress proteins according to the invention may be prepared by a variety of processes, using an appropriate bacteria cell culture, e.g. a C.jejikeium cell culture, as starting material. Thus, for example, suitable bacterial cells may be harvested, lysed and, after separation of the cell debris the resulting cell extract may be fractionated using conventional separation techniques such as ion exchange and gel chromatography and electrophoresis, and immunopurification methods, for example affinity chromatography using an antibody capable of recognising the protein, for example an antibody capable of recognising the peptide KVIRKNIVKKMIE. During the preparation the presence of the desired protein may be monitored using any appropriate standard analytical technique based, for example on an antibody capable of recognising an epitope on the protein, for example an antibody capable of recognising the peptide KVIRKNIVKKMIE. Use of the above techniques can be expected to yield the stress protein in a purified form.

Alternatively, the protein may be cloned and expressed starting from an appropriate C.jejikeium genomic library and using standard screening and recombinant DNA techniques.

Brief Description of the Drawings

In the follow description various embodiments of the present invention are described with reference to the accompanying drawings in which:

- Figure 1 - shows sequential immunoblots of the IgG response to a C.jeikeyum septicaemia in a patient who survived the infection.
- Figure 2 - shows an immunoblot of the IgM and IgG response to a C.jeikeyum endocarditis in a patient who survived the infection.
- Figures 3 and 4 - each shows an immunoblot of a C.jeikeyum isolate probed with rabbit antisera raised against a pressate of C.albicans and against the peptide LKVIRKNIVKKMIE-Cys, and a monoclonal antibody raised against the same peptide, both before and after cross-absorption with peptide LKVIRKNIVKKMIE

The following Examples illustrate the invention.

Example 1

The following reports the results of immunoblotting sera from patients with a C.jeikeyum septicaemia or endocarditis and compares the data with immunoblots obtained from sera which were negative for the JK coryneform. [Jackman, P.J.H. and Pelezynska, J., J. Gen. Microbiology (1986), 132, 1911-1915].

Patients

Controls - Sera were examined from 18 pyrexial neutropenic leukaemics where all cultures were negative for the JK coryneform. This included blood and those taken from any indwelling intravenous line.

Sepicaemias

Between two and five sequential sera were examined from 14 patients with C.jeiikeium septicaemias. Thirteen of the 14 cases came from neutropenic patients who recovered on vancomycin therapy. Their pyrexia correspond with a blood culture positive with the C.jeiikeium. Three of the cases had acute lymphatic leukaemia and the remaining 10 had acute myeloid leukaemia. The 14th case came from a patient who was admitted to an Intensive Therapy Unit following a road traffic accident. He was not neutropenic and his C.jeiikeium septicaemia followed colonization of a long-term indwelling central line. He responded to vancomycin therapy.

Endocarditis

Case 15 came from a patient with ulcerative colitis with an intravenous feeding line which became colonized by C.jeiikeium. The patient subsequently developed endocarditis as judged by a positive echo-cardiogram and continuing pyrexia. Three sets of blood cultures grew the C.jeiikeium and the patient gradually recovered after systemic vancomycin therapy.

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Strain

A clinical isolate of the C.jeiikeium obtained from a septicaemic case was used throughout.

Preparation of Protein Extracts

The C.jeiikeium isolate was sub-cultured onto Columbia blood-agar (Oxoid) and incubated at 35°C for 48 hours aerobically. The plates were harvested with a loop in distilled water and the resulting cell suspension spun at 6,000g for 20 minutes. The pellet was re-suspended in its own volume of sterile distilled water and placed inside an Xpress. It was crushed at -20°C and centrifuged at 12,000G for 20 minutes. The supernatant was used in subsequent experiments and stored at -20°C. It was standardised to a protein concentration of 10mg/ml.

Immunoblotting of Patients Sera

After heating with cracking buffer [2.6% sodium dodecyl sulphate, 1.3% 2-mercapto-ethanol, 6% glycerol, 0.2% bromophenol blue, 0.05% M Tris hydrochloride (pH 6.8)] at 10°C for 2 minutes, 30µg of the C.jeiikeium pressate was loaded onto each well of a 10% polyacrylamide gel. Electrophoresis and transblotting were performed as described by Matthews, R.C. et al, [Lancet, ii 1415-1418 (1984); and J. Clin. Microbiol. 25, 230-237 (1987)]. The gel was transferred onto a nitrocellulose membrane in an LKB transblotter (LKB laboratories). The buffer contained methanol (20%), 25mM Tris and 192mM glycine at pH 8.3 and transfer was allowed to proceed at 25°C for 45 minutes. The nitrocellulose paper was blocked in bovine-serum albumen (3%) in buffered saline (NaCl 0.9% and 10mM Tris,

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pH 7.4) at 4°C overnight. The nitrocellulose was then incubated at 35°C for 2 hours with the patients serum diluted 1:10 in buffered saline containing bovine serum albumin (3%) and Tween 20 (0.05%). After washing 5 times for 30 minutes in saline (0.9%) and Tween 20 (0.05%), the nitrocellulose was incubated for one hour at 35°C with alkaline phosphatase conjugated goat anti-human immunoglobulin M (IgM) or IgG (Sigma Chemical Co.). After washing again, the membranes were incubated for 15 minutes at 25°C with buffer (100mM Tris hydrochloride pH 9.5, 100mM NaCl, 5mM MgCl₂) containing a mixture of 66µl/per 10mls of nitro-blue tetrazolium (NBT 50 mg per ml in N,N-dimethylformamide 70%) and 33µg per 10mls of 5-bromo-4-chloro-3 indolylphosphate (BCIP 50 mg/ml in N,N-dimethylformide 70%). The reaction was stopped by washing in water. All the immunoblots were examined and split into trace responses, [where a reflectance densitometer produced a trace with a height of less than 40mm] and positive responses [where the height was greater than 40mm].

Molecular weight markers were Prestained Rainbow Markers (code RPN.756 Amersham International). These were myosin, 200 kDa; phosphorylase b, 92.5 kDa; bovine serum albumin, 69 kDa; ovalbumin, 46kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.3 kDa.

RESULTS

The results from the 18 control patients are given in Table 1. Six bands were detected and the commonest antibody response was to the band at 110 kDa. Sequential sera were available in four of the patients where there was an antibody response and the patterns did not change

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over a period of at least two weeks. Trace and positive results [see above] were combined for this Table.

Apparent
Molecular
Weight

(kDa)	IgM	IgG
170	3	3
154	0	1
110	4	3
86	0	1
70	0	4
60	1	0

Table 1: Details of the antibody responses in control patients.

Septicaemic Patients

The results from these are summarised in Tables 2 and 3. They are given according to four criteria. These are: a constant trace reaction, a constant positive reaction, an increase in brightness which is at least a doubling of intensity as measured by reflectance densitometry and finally the appearance of a new antigenic band. The results from the serum taken prior to C.jejikeium infection were compared to those after infection. IgM levels (Table 2) were static for the bands at 154, 86, 80, 70, 65, 60 and 43 kDa. A maximum of two patients showed changes in the bands at 170, 160, 158, 148 and 57 kDa. An increase in or the appearance of IgM against the bands at 110, 52 and 50 kDa were the most marked changes which occurred during infection. IgG levels (Table 3) showed a similar

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picture to IgM. Twelve patients developed a more pronounced antibody response to the band at 110 kDa. All the patients upon recovery produced IgG against the band at 52 kDa and an example of this is illustrated in Figure 1. This patient was the road traffic accident and also showed an increase in the serum level of IgG against the band at 110 kDa. Tract 1 is prior to septicaemia whilst tracks 2 and 3 were taken, respectively, just after the cessation of vancomycin therapy and five days later. Nine of the 14 patients also produced IgG against the 50 kDa band.

The case of endocarditis (Figure 2) had IgM and IgG against all the antigenic bands previously described for the septicaemic patients. The IgM response faded on successful treatment whilst the IgG increased to the 50 and 52 kDa bands.

Summary

The above results show that recovery from the septicaemia was associated with production of both IgM and IgG against antigenic bands of 50, 52 and 110 KDa. Antibody against the 110 KDa band was present in controls but the antibody against the 50 and 52 KDa bands was specific to those patients who had on-going or previous C.jejikeium infection. In the case of the C.jejikeium endocarditis recovery was also associated with sero-conversion to the 50 and 52 KDa bands. This illustrates the potential of using either of these antigens as the basis of a serodiagnostic test.

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Apparent Molecular Weight (kDa)	Constant Trace	IgM Positive	Increase in brightness	Appearance of band
170	4	8	2	
160	2	2	1	1
158	3	1	1	1
154	3	1		
148		2	1	
110	3	3	5	3
86		1		
80		1		
70		2		
65		2		
60				
57	1			1
52	2			7
50	1			3
43	1			
40				

Table 2: Details of the IgM response in septicaemic patients to individual C.jejikeium bands.

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Apparent Molecular Weight (kDa)	Constant Trace	IgG Positive	Increase in brightness	Appearance of band
170	5	5	2	1
160	1	5	2	1
158	2	2	1	1
154	2	2		
148	1	1		
110		2	7	5
86	3	2		
80	3	3		
70	4	2	2	
65	3		2	1
60	1			
57	1	1		
52				14
50				9
43	1	1		
40	1	1		

Table 3: Details of IgG response in septicaemic patients to individual C.jejikeium bands.

Example 2

Rabbit hyperimmune antisera against the peptide LKVIRKNIVKKMIE-Cys and against a pressate of C.albicans were raised using conventional methods [see for example Burnie, J., J. Immunol. Meth. (1985), 82, 267-280].

A murine monoclonal antibody was raised against LKVIRKNIVKKMIE-Cys-KLH. Balb/c and CBA x Balb/c F1 mice

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were injected subcutaneously with 50 μ g of immunogen in sterile complete Freund's Adjuvant and thereafter at intervals of 14 days, intraperitoneally with 50 μ g immunogen in Incomplete Freund's Adjuvant until seroconversion.

Fusion was performed 4 days after a final immunisation of 50 μ g immunogen intravenously in sterile physiological saline. Fusion, hybridoma screening, clonal selection and antibody analysis were performed according to standard protocols, essentially as described by de St. Groth S.F. and Scheidegger D., J. Immunol. Methods 35, 1-21 (1980). Selected hybridomas were screened for activity against a C.albicans 47Kd antigen by immunoblotting against C.albicans. Positive hybridomas were re-cloned and re-assayed.

Characterisation of monoclonal antibody

A novel hybridoma cell line (CA-STR7-1) obtained according to the above methods produced a monoclonal antibody which recognised both the C.albicans 47Kd antigen and the antigen of approximately 92Kd on immunoblots of C.albicans grown at 37°C, in both the yeast and mycelial forms. At 23°C the 47Kd antigen was visible but not the 92Kd antigen with this monoclonal antibody.

Immunoblotting

The above rabbit antisera and monoclonal antibody were used to probe the C.jejikeium isolate (see Example 1).

Figures 3 and 4 show immunoblots (see method in Example 1) obtained both before and after cross-absorption with the peptide KVIRKNIVKKMIE [1 mg/ml as follows]:

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Figure 3

- | | |
|---------|--|
| Tract 1 | Monoclonal against
LKVIRKNIVKKMIE-Cys
crossreacting with 52Kd band |
| Tract 2 | Monoclonal against
LKVIRKNIVKKMIE-Cys after
crossabsorption (no reaction) |
| Tract 3 | Rabbit hyperimmune antiserum
against LKVIRKNIVKKMIE-Cys
crossreacting with 52Kd band |
| Tract 4 | Rabbit hyperimmune antiserum
against LKVIRKNIVKKMIE-Cys
after crossabsorption (no
reaction) |
| Tract 5 | Rabbit polyclonal hyperimmune
against a pressate of
<u>C.albicans</u> crossreacting with
52Kd band. |

Figure 4

- | | |
|---------|--|
| Tract 1 | Rabbit hyperimmune antiserum
against LKVIRKNIVKKMIE-Cys
after crossabsorption (no
reaction with 86Kd band). |
| Tract 2 | Rabbit hyperimmune antiserum
against LKVIRKNIVKKMIE-Cys
crossreacting wioth 86KDa band. |
| Tract 3 | Mouse monoclonal against
LKVIRKNIVKKMIE-Cys (no reaction) |

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Tract 4 Mouse monoclonal against
 LKVIRNIVKKMIE-Cys after
 crossabsorption (no reaction)

Summary

The antisera and monoclonal antibody each detected the C.jeiikeium, illustrating their potential for use in a diagnostic test.

The rabbit hyperimmune serum crossreacted with bands at 86Kd and 52Kd. These were both neutralised by the peptide LKVIRKNIVKKMIE at 1mg/ml. The monoclonal antibody reacted with the 52Kd band and was neutralised by the corresponding peptide. The rabbit hyperimmune serum against a pressate of C.albicans crossreacted with the 52Kd band.

CLAIMS

1. A bacterial stress protein having an apparent molecular weight of around 86 Kd or a fragment or an analogue thereof.
2. A stress protein according to Claim 1 obtainable from a Gram-positive bacterial strain.
3. A stress protein according to Claim 2 wherein the Gram-positive strain is a strain belonging to the genus *Corynebacterium*.
4. A stress protein according to Claim 3 wherein the strain belonging to the genus *Corynebacterium* is a strain of *Corynebacterium diphtheriae* or *Corynebacterium jeikeium* or a related Coryneform species.
5. A stress protein according to any of Claims 1 to 4 which has one or more of the following features:
 - (1) it is an immunodominant conserved antigen;
 - (2) patients with *C. jeikeium* septicaemia on recovery have antibody to a 52 Kd breakdown product of the stress protein; and
 - (3) patients with *C. jeikeium* endocarditis have antibody to a 52 Kd breakdown product of the stress protein;

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- (4) it cross-reacts with a peptide antigen KVIRKNIVKKMIE using mouse monoclonal antibody against the peptide antigen; and
 - (5) cross-reaction of the stress protein and mouse monoclonal antibody against the peptide antigen KVIRKNIVKKMIE is neutralised by peptide KVIRKNIVKKMIE.
- 6. A fragment of a stress protein according to any one of the preceding claims which has an apparent molecular weight of around 52 Kd or 50 Kd.
 - 7. A stress protein or fragment according to any one of the preceding claims for use in a diagnostic test for bacterial infection.
 - 8. A stress protein or fragment according to Claim 7 wherein the diagnostic test is an enzyme-linked immunosorbent assay, a radioimmunoassay or a latex agglutination assay.
 - 9. A stress protein or fragment according to any one of Claims 1 to 6 for use as an immunogen.
 - 10. An inhibitor of a bacterial stress protein having an apparent molecular weight of 86 Kd or a fragment or an analogue thereof, for use in the diagnosis or treatment of bacterial infections.
 - 11. An antibody specific for one or more epitopes of a bacterial stress protein having an apparent molecular weight of 86 Kd or a fragment or an analogue thereof, for use in the diagnosis or treatment of bacterial infections.

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12. An antibody according to Claim 11 which is capable of recognising the peptide KVIRKNIVKKMIE.
13. An antibody according to Claims 11 or 12 which is a monoclonal antibody.
14. An antibody according to any one of Claims 11 to 13 for use in the diagnosis or treatment of an infection due to a Gram-positive organism.
15. An antibody according to Claim 14 for use in the diagnosis or treatment of an infection due to a Coryneform organism.
16. An antibody according to Claim 15 for use in the diagnosis or treatment of an infection due to a C.diphtheriae or C.jejikeium organism.

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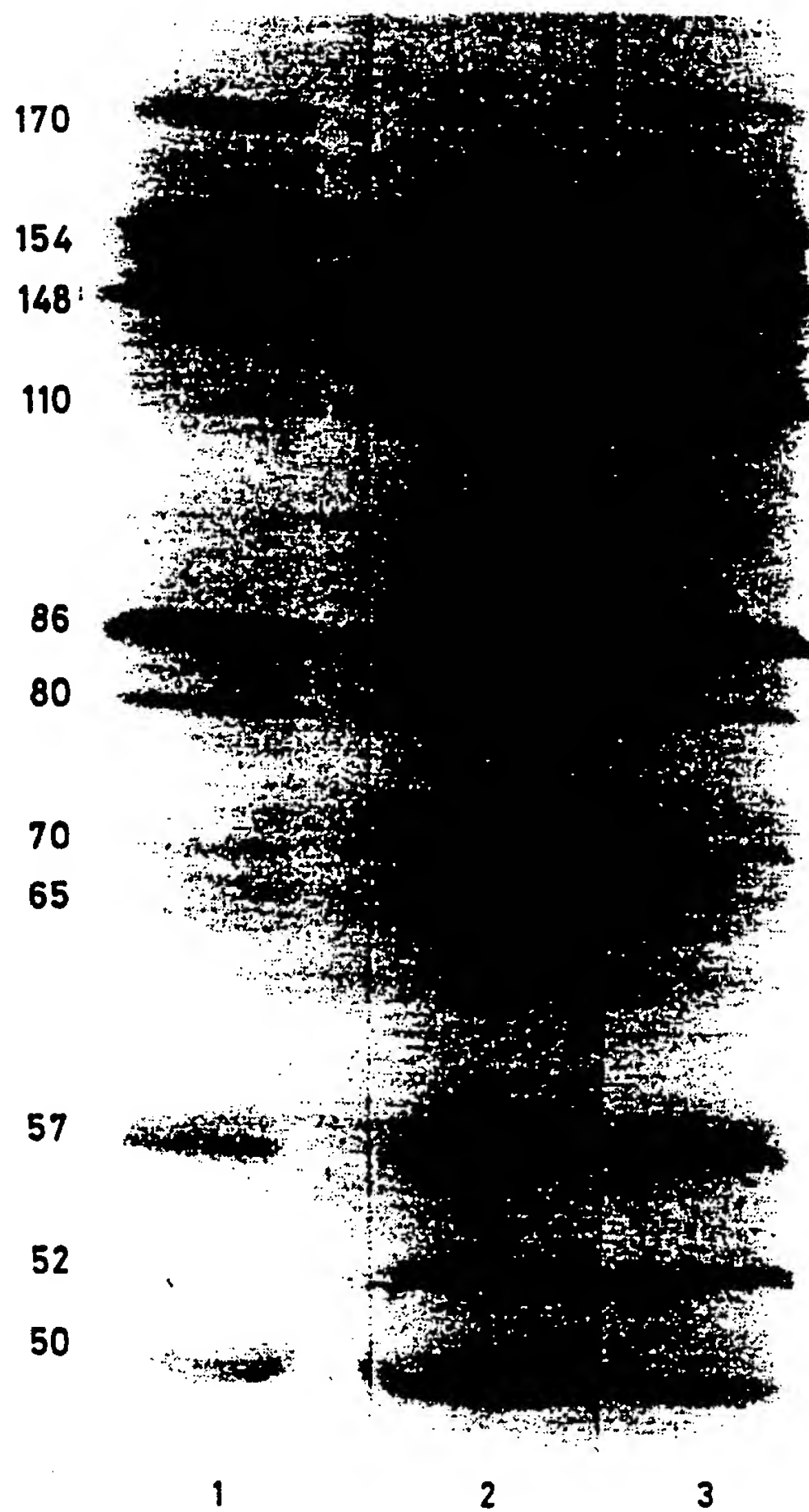


Fig. 1

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Fig. 2

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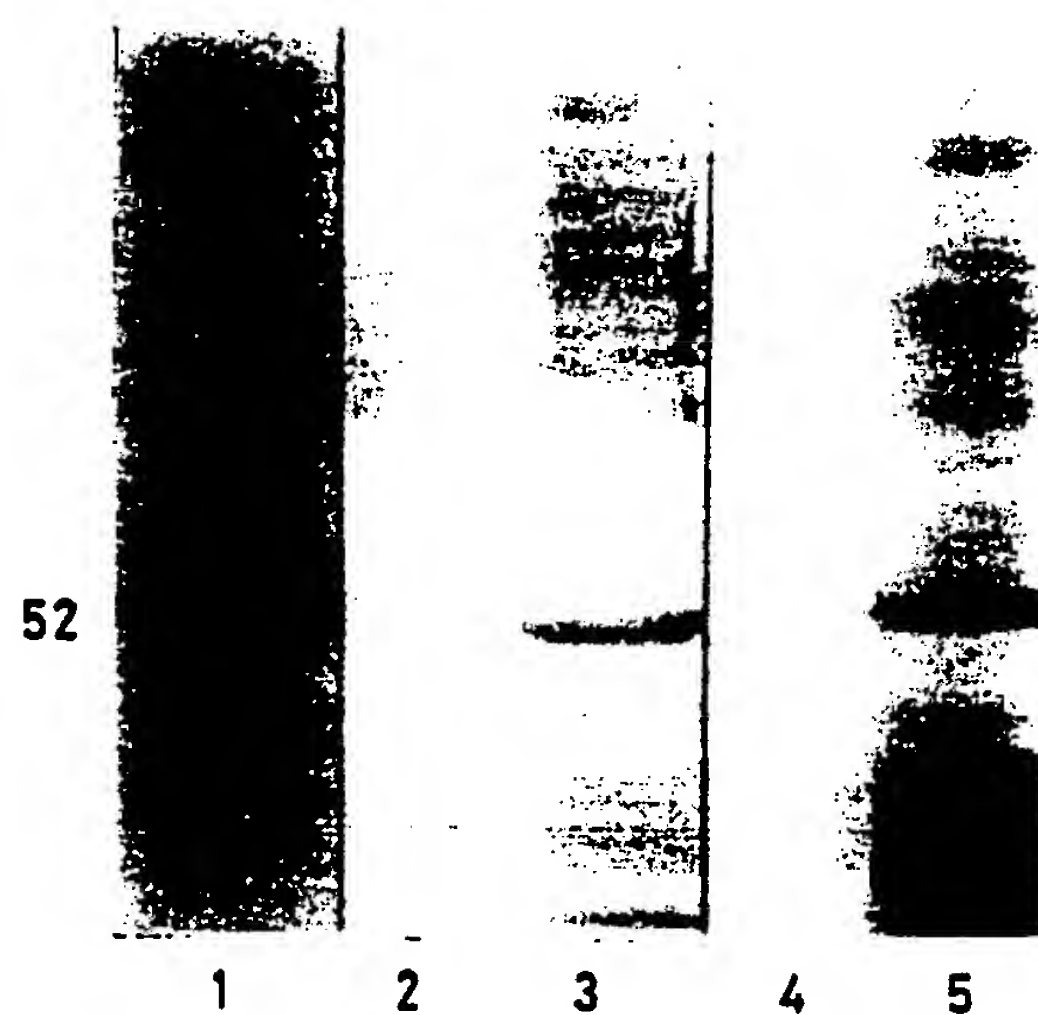


Fig. 3

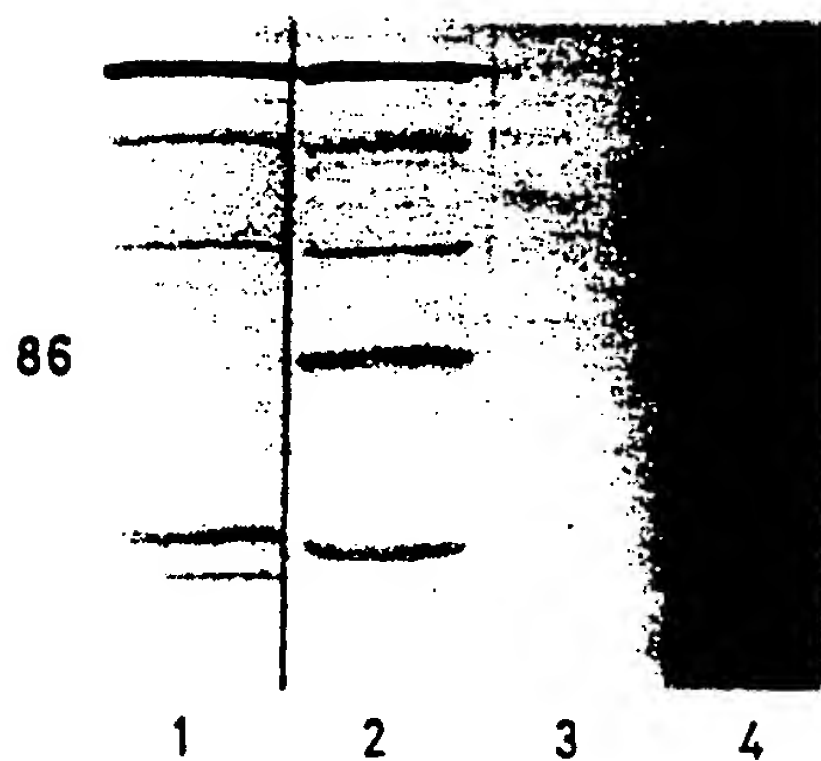


Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 91/01252

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 07 K 15/00 C 12 P 21/08 A 61 K 37/02
A 61 K 39/395 G 01 N 33/569

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
Int.Cl.5	C 07 K C 12 P A 61 K G 01 N

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
A	Chemical Abstracts, volume 111, no. 13, 25 September 1989, (Columbus, Ohio, US) R. Matthews et al.: "Cloning of a DNA sequence encoding a major fragment of the 47 kilodalton stress protein homologue of Candida albicans", see page 199, abstract 110265h, & FEMS Microbiol. Lett. 1989, 60(1), 25-30 ---	1-9
A	Biological Abstracts, volume 90, 1990 (Phil. P.A., US) H. Yamaguchi et al.: "Yersinia enterocolitica immunodominant 60 kDa antigen, common to a broad range of bacteria, is a heat-shock protein", see abstract 54993, & J. GEN. MICROBIOL 136(6): 1091-1098, 1990 --- -/-	1-16

¹⁰ Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

29-10-1991

Date of Mailing of this International Search Report

25. 11. 91

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

Nicole De Bie



III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	Infection and Immunity, volume 58, no. 5, May 1990, American Society for Microbiology, (Washington, DC 20005) C.K. Stover et al.: "Molecular cloning and sequence analysis of the Sta58 major antigen gene of rickettsia tsutsugamushi: sequence homology and antigenic comparison of Sta58 to the 60-kilodalton family of stress proteins", pages 1360-1368, see the whole article ---	1-16
A	Proceedings of the National Academy of Sciences, volume 87, no. 3, February 1990, Biochemistry, J.L. Brisette et al.: "Phage shock protein, a stress protein of Escherichia coli", pages 862-866, see the whole article -----	1-16